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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07H 21/00, 21/04, A61K 48/00

A1

(11) International Publication Number: WO 95/17414

(43) International Publication Date: 29 June 1995 (29.06.95)

(21) International Application Number: PCT/US94/13687

(22) International Filing Date: 1 December 1994 (01.12.94)

(30) Priority Data:
08/172,538 23 December 1993 (23.12.93) US

(71) Applicant: APOLLON, INC. [US/US]; One Great Valley Parkway, Malvern, PA 19355-1423 (US).

(72) Inventors: CONEY, Leslie, R.; Apartment A201, 1000 Conestoga Road, Rosemont, PA 19010 (US). PACHUK, Catherine, J.; Apartment B511, 223 Scottsdale Road, Lansdowne, PA 19050 (US). YOON, Kyonggeun; 802 Llanelly Lane, Berwyn, PA 19312 (US).

(74) Agent: FRASER, Janis, K.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ANTI-HEPATITIS B VIRAL OLIGONUCLEOTIDES

(57) Abstract

The invention relates to methods and compositions for inhibition of viral replication. In particular, complete and irreversible termination of replication of hepatitis B virus is achieved by introducing into a target cell an oligonucleotide complementary to HBV plus strand and 5' and 3' flanking nucleotides.

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ANTI-HEPATITIS B VIRAL OLIGONUCLEOTIDES

Field of Invention

The invention relates to oligonucleotide compositions, pharmaceutical compositions containing such oligonucleotides, and their use for the prevention and treatment of hepatitis B infection.

Background of the Invention

The consequences of a viral infection depend upon a number of factors, both viral and host. Those factors which affect pathogenesis include the number of infecting viral particles and their path to susceptible cells, the speed of viral multiplication and spread, the effect of the virus on cell functions, the host's secondary responses to the cellular injury, and the immunologic and non-specific defenses of the host. In general, the effects of viral infection include acute and chronic clinical diseases, asymptomatic infections, induction of various cancers, and chronic progressive neurological disorders. Viruses are potent infectious pathogenic agents because virions produced in one cell can invade other cells and thus cause a spreading infection. Viruses cause important functional alterations of the invaded cells, often resulting in death of the cells.

Hepatitis viruses constitute a major medical problem throughout the world. Like the other hepatitis viruses, the hepatitis B virus (HBV) produces a whole spectrum of illnesses, ranging from acute to chronic and from subclinical or asymptomatic to fatal and fulminant. Approximately 5% of the world's population, probably at least 400 million people, are presently infected with the hepatitis B virus (HBV). HBV presents a high risk of acute fulminant hepatitis, as well as chronic liver

disease, including cirrhosis, chronic active hepatitis, and the eventual development of primary hepatocellular carcinoma in individuals who remain chronic carriers of the virus.

Therapeutic studies during the last ten years have identified promising drugs with antiviral effects, including the nucleotide analog adenine arabinoside (Ara-A), its more soluble monophosphate Ara-AMP, and Interferon-alpha. Although effective in some patients, treatment with such agents has been shown frequently to result in only a transient response, or to have significant toxicity. Accordingly, there is a continuing need for methods and therapeutic agents to stop viral replication and prevent the spread of the virus to additional cells. However, this goal presents considerable difficulties. A major problem is that of inhibiting the virus without harming the host cells. The dependence of viral multiplication on cellular genes limits the points of differential attack. Even the largest viruses have few biochemical reactions that are not duplicative of those of the host. Further, it is only after extensive viral multiplication and cellular alteration have occurred that viral infections become evident. Therefore, the usual approach to control is prophylaxis. Therapy in most cases is limited to situations where the killing of some uninfected cells can be tolerated if the damage is subsequently repaired.

Another important limitation of antiviral therapy is the emergence of resistant mutants. In order to minimize selection of such mutants, the principles valid for treatment of bacterial infections are equally applicable to viruses: adequate dosage, multi-drug treatment, and avoiding therapy unless clearly indicated. Therefore, because of the serious nature of viral infection and the obstacles presented by the nature of

the infecting virus, there is an urgent need for methods which control viral replication. A method which would be applicable to RNA and DNA viruses would have widespread applicability.

Synthetic antisense oligonucleotides have been used as inhibitors of viral gene expression. Smith et al., *Proc. Natl. Acad. Sci. USA* 83:2787-2791 (1986), report antiviral activity of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type I immediate early pre-mRNAs 4 and 5. See also Agris *et al.*, Inhibition of vesicular stomatitis virus protein synthesis and infection by methylphosphonates, *Biochem. 25*, 6268-6275 (1986); Zamecnik *et al.*, Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide, *Proc. Natl. Acad. Sci. USA* 75:280-284 (1978); Zamecnik *et al.*, Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA, *Proc. Natl. Acad. Sci. USA 83*, 4143-4146 (1986). Goodarzi *et al.*, *J. Gen. Virol. 71*:3021-3025 (1990), report inhibition of the expression of the gene for hepatitis B virus surface antigen by antisense oligodeoxynucleotides directed at the cap site of mRNA and regions of the translational initiation site of the HBsAg gene. Offensperger et al., *In vivo* inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides, *EMBO J. 12*: 1257-1262, No. 3 (1993), report inhibition of duck hepatitis B virus (DHBV) replication by antisense oligdeoxynucleotides from the pre-S/S-region, the polymerase region, and the pre-C/C region. Particularly effective were an antisense oligodeoxynucleotide from the pre-S region and one from the direct repeat II (DRII) region.

SUMMARY OF THE INVENTION

The invention relates to antisense oligonucleotides, preferably antisense oligodeoxynucleotides, as antiviral agents against HBV; pharmaceutical compositions providing such antiviral oligonucleotides; and methods for their use in inhibiting HBV. Antisense oligonucleotide compositions complementary to the HBV DR2 region completely block viral transcription, antigen production, and replication. Such antiviral oligonucleotides can be provided to the target cell either exogenously as an antisense DNA or RNA, or by insertion of a sense DNA sequence into an expression vector capable of producing multiple copies of the antisense oligonucleotides endogenously within the target cell.

The invention includes an oligonucleotide having antiviral activity against hepatitis B virus (HBV), consisting essentially of a sequence substantially complementary to a portion of plus (+) strand of the HBV genome, which portion consists of the DR2 11-mer (SEQ ID NO: 44) plus 0-6 nucleotides of 5′ flanking sequence and 0-30 nucleotides of 3′ flanking sequence. Examples of such oligonucleotides include those with the sequence:
5′-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 45),
5′-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 46),
5′-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 47), or
5′-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 48),
or a portion thereof, which portion is preferably at least 15 nucleotides (and more preferably at least 18 nucleotides) in length. The oligonucleotide preferably includes the sequence 5′-ACGTGCAGAGGTGAAGCG-3′ (SEQ ID

NO: 21). Examples of oligonucleotides of the invention include the following:

5'-CAACGTGCAGAGGTGAAGCGA-3' (OLIGO HBV 10011; SEQ ID NO: 6);

5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);

5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);

5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);

5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);

5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);

5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51);

5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52);

5'-CGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 141: SEQ ID NO: 25);

5'-GTGCAGAGGTGAAGCGA-3' (OLIGO CJP 142: SEQ ID NO: 26);

5'-TGCAGAGGTAAGCGA-3' (OLIGO CJP 143: SEQ ID NO: 27);

5'-GCAGAGGTGAAGCGA-3' (OLIGO CJP 144: SEQ ID NO: 28);

5'-CAGAGGTGAAGCGA-3' (OLIGO CJP 145: SEQ ID NO: 29);

5'-AGAGGTGAAGCGA-3' (OLIGO CJP 146: SEQ ID NO: 30);

5'-GAGGTGAAGCGA-3' (OLIGO CJP 147: SEQ ID NO: 31);

5'-AGGTGAAGCGA-3' (OLIGO CJP 148: SEQ ID NO: 32);

5'-CGACGTGCAGAGGTGAAGC-3' (OLIGO CJP 149: SEQ ID NO: 33);

5'-CGACGTGCAGAGGTGAAG-3' (OLIGO CJP 151: SEQ ID NO: 34);

5'-CGACGTGCAGAGGTGAA-3' (OLIGO CJP 152: SEQ ID NO: 35);

5'-CGACGTGCAGAGGTGA-3' (OLIGO CJP 153; SEQ ID NO: 36);

5'-CGACGTGCAGAGGTG-3' (OLIGO CJP 154: SEQ ID NO: 37);

5'-CGACGTGCAGAGGT-3' (OLIGO CJP 155: SEQ ID NO: 38);

5'-CGACGTGCAGAGG-3' (OLIGO CJP 156: SEQ ID NO: 39);

5'-CGACGTGCAGAG-3' (OLIGO CJP 157: SEQ ID NO: 40);

5'-AACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 158: SEQ ID NO: 41);

5'-CGACGTGCAGAGGTGAAGCGAAG-3' (OLIGO CJP 159: SEQ ID NO: 42); and

5'-CGACGTGCAGAGGTGAAGCGAA-3' (OLIGO CJP 160: SEQ ID NO: 43).

Preferred oligonucleotides of the invention include those having a sequence consisting essentially of one of the following:

5'-CAACGTGCAGAGGTGAAGCGA-3' (OLIGO HBV 10011; SEQ ID NO: 6);

5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);

5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);

5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);

5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);

5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);

5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51); or

5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52).

The oligonucleotides of the invention can be used in a method of preventing replication of HBV in a mammalian cell, which method includes the step of introducing into the cell an inhibitory amount of (a) the oligonucleotide, or (b) an expression vector containing a sequence that is transcribed within the cell to generate an oligoribonucleotide of the invention. Such an expression vector would preferably include transcription control sequences that permit it to be expressed within a hepatocyte. The oligonucleotide may be administered to an animal in the form of a pharmaceutical composition consisting essentially of an amount of the oligonucleotide effective to inhibit replication of HBV in the liver cells of an animal, and a pharmaceutically acceptable carrier.

It is understood that when the oligonucleotide of the invention is a ribonucleotide, "T" in each of the sequences set forth herein represents "U".

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a map of the HBV genome, showing the coding organization of the four major open reading frames, as well as the 3.5 kb pregenomic RNA and the 2.1 kb subgenomic RNA species. DR1 and DR2 are shown.

Fig. 2 is a representation of the computer-generated putative secondary structure of the RNA sequence of HBV (HPBADW1) (n.t.s. 1500 to n.t.s. 1700), showing DR2 as part of a stem-loop structure.

DETAILED DESCRIPTION

Human hepatitis B virus (HBV) is now recognized to be a member of a family of animal viruses called hepadnaviruses (hepatotropic DNA viruses). Human HBV is classified as a hepadnavirus type 1. Similar viruses infect other animal species, including woodchucks, ground and tree squirrels, Pekin duck, and heron, producing acute and chronic hepatitis as well as hepatocellular carcinoma. Full-length molecular clones of these hepadnaviruses have been obtained and their nucleotide sequences determined. The coding organization of the mammalian viruses is now known to be virtually identical to that of human HBV, while the avian viruses are more divergent in terms of nucleotide sequence, biological properties, and coding organization. The duck HBV (DHBV) genome appears to lack an X region, and its core antigen coding region is substantially larger that that of the mammalian viruses. Ganem & Varmus, The Molecular Biology of the Hepatitis B Viruses; *Ann. Rev. Biochem. 56*:651-93 (1987).

Replication strategy of the hepatitis B virus is discussed by Seeger *et al.*, *Science 232*:477-484 (1986); Khudyakov *et al.*, *FEBS Letters 243*:115-118 (1989); Will *et al.*, *J. of Virol. 61*:904-911 (1987); and Hirsch *et al.*, *Nature 344*:552-555 (1990).

Infectious human HBV virions, called "Dane particles", are 42 or 43 nm double-shelled particles which include the outer coat of HBV surface antigen (HBsAg) and the HBV core antigen (HBcAg), a basic phosphoprotein of 21 kd. Within the HBV nucleocapsid core is a predominantly double-stranded, but partially single stranded, DNA genome measuring 3200 base pairs, as well as an endogenous DNA polymerase which directs replication and repair of HBV DNA. The full-length strand of HBV DNA is complementary to the viral RNAs, including the mRNAs, and by convention is designated to be of minus polarity. The shorter complementary strand is designated the plus strand. While the 5′ end of the plus strand is fixed, the position of the 3′ end is variable, even within molecules of the same viral stock. In the endogenous polymerase reaction, the single-stranded gap is repaired by the addition of nucleotides to the 3′ end of the plus strand DNA. A second asymmetry occurs at the 5′ termini of the viral genome's two strands: protein is covalently linked to the 5′ end of the minus strand, whereas an oligonucleotide is attached to the 5′ end of the plus strand.

Replication of HBV proceeds via reverse transcription of an RNA intermediate using protein and RNA primers for the generation of the first and second DNA strands. Large sections of the genome are translated in more than one reading frame. Within a given reading frame, multiple proteins are expressed from overlapping transcripts, using different in-phase initiator codons. The resulting closely related gene products are posttranslationally processed and assembled into a variety of structures of differing function or subcellular distribution.

Four major open reading frames (ORFs) encoded by the HBV minus strand have been identified and

characterized: 1) the pre-S and S gene, which code for the HBsAg and several other less well characterized gene products; 2) the C gene, which codes for HBcAg and HBeAg; 3) the P gene, which codes for the viral DNA polymerase; and 4) the X gene, which codes for the transactivating X protein, HBx, often observed in patients with hepatocellular carcinoma. (HBeAg results from proteolytic cleavage of the p22 precore intermediate, and is secreted from the cell. It is found in serum as a 17 kd protein.)

Four major steps are believed to be fundamental to the replication of hepadnavirus genomes:

1. closing of the single-stranded gap by the addition of nucleotides to the 3′ end of the plus strand DNA, to form covalently closed circular DNA (cccDNA) within the nucleus of infected hepatocytes;

2. transcription of cccDNA by host RNA polymerase to generate an RNA template of plus strand polarity for reverse transcription, with encapsidation of the pre-genomic RNA into viral cores;

3. synthesis of the first (minus) strand of DNA by copying pregenomic RNA, using a protein primer (this step is termed core associated reverse transcription); and

4. synthesis of the second (plus) strand of DNA by copying the first DNA strand using an oligomer of viral RNA as primer, to form the mature viral genomic DNA. Amplification of the viral genome is believed to occur during synthesis of pregenomic RNA from cccDNA.

HBV viral RNA serves as both the template for synthesis of genomic DNA via reverse transcription and the messenger RNA for synthesis of certain viral proteins. This is achieved by the synthesis of two classes of viral RNA, genomic (3.5 kb in length, containing the complete viral genetic information) and

subgenomic (2.1 and 2.4 kb in length). All of these RNAs are of plus strand polarity, unspliced, and polyadenylated at a common 3′ terminus. Within the HBV genome are conserved *cis*-acting elements that play important roles in the life cycle of the virus. Chief among these are 11-nucleotide "direct repeat" sequence designated DR1 and DR2. Dr1 and DR2 are distinguished from each other by their positions in the genome, their flanking sequences, and their biological functions. DR1 and DR2 are located near the 5′ and the 3′ ends of the HBV plus strand, respectively, and play critical roles in the initiation of viral DNA synthesis. In the pregenomic RNA, there are two copies of DR1, found at the 3′ and 5′ ends, respectively, and one copy of DR2 located near the 3′ end.

The other major conserved sequence is the element TATAAA (SEQ ID NO: 1) found within the 5′ end of the core antigen coding sequence, which forms part of the cleavage/polyadenylation signal specifying the common 3′ termini of viral mRNAs. For details of the structure and function of the DR1 and DR2 sequences, see Ganem & Varmus, The Molecular Biology of the Hepatitis B Viruses, *Ann. Rev. Biochem. 56*:651-93 (1987), and Seeger, Ganem, and Varmus, *Science 232*:477-484 (1986), the teachings of which are hereby incorporated by reference. See also Figure 1, which sets forth a map of the HBV viral genome, showing the organizational structure of the four major ORFs and the pregenomic and subgenomic RNA species.

Synthesis of the minus strand of viral DNA is believed to begin with the DR1 sequence that resides within the terminal repeat region, R, found at both the 5′ and 3′ ends of the pregenomic RNA. Initiation could occur near either the 5′ or the 3′ end of the RNA template, since DR1 resides within the R sequence that is terminally repeated in pregenomic RNA. As discussed in

Ganem & Varmus, *supra*, at 663-664, initiation at the 3′ end would permit elongation without interruption across the entire genomic sequence, ending with a second copy of the 9 nucleotide sequence found between the DR1 initiation site and the 5′ boundary of R, whereas initiation at the 5′ end would require transfer of the growing minus strand DNA molecule to the 3′ end of the same or another pregenomic RNA molecule.

The initiation site for synthesis of plus strand DNA occurs on a minus strand DNA template at the sequence which is complementary to DR2 (DR2′). A short oligomer of viral RNA, a fragment of the pregenomic RNA generated by RNAse H, is covalently linked to the DNA at this site. However, when the attached RNA was sequenced (Lien et al. *J. Virol.* 57:229-37 (1986)), the expected DR sequence was found to be flanked by 6 nucleotides from the DR1 region, rather than the expected DR2 region. This suggests that an oligomer containing DR1, from either the 5′ or 3′ copy of R, basepairs with the DR2′ site in the DNA minus strand, and serves as a primer for initiation of plus strand synthesis from that position. The fact that the oligomer has a 5′ cap structure is evidence that the plus strand primer originates from the 5′ end of the pregenomic RNA, although the reason for such a complicated priming mechanism is not immediately clear. Synthesis of the plus strand reaches a roadblock at the protein-linked 5′ end of the minus strand template, requiring transfer of the partial plus strand to the 3′ end of a minus strand template. For unknown reasons, the plus strand is usually not extended to the full length of the minus strand; instead, plus strands are incomplete and heterogeneous in length, with open circles the dominant form of virion DNA.

The nucleotide sequence of DR2 and flanking sequences both 5′ and 3′ to DR2 are highly conserved

among various HBV HBsAg subtypes and strains. The nucleotide sequence of the DR2 region of HBV HBsAg subtype ADW was compared with corresponding sequences from a number of other strains or subtypes of HBV by using sequence data available in GenBank. Sequences and designations are as listed in GenBank®. See Table I below, where the symbol ":" indicates a nucleotide identical to the corresponding position of HPBADW. According to GenBank, DR2 of HPBADW (Hepatitis B Virus Subtype ADW) consists of nucleotides 1592 through 1602, having the sequence TTCACCTCTGC (SEQ ID NO: 2). The numbering listed in Table 1 is that of Hepatitis B Virus Subtype ADW from GenBank. The nucleotide sequences of various hepatitis virus strains can be found in Okamoto *et al.*, *J. Gen. Virol.* *69*:2575-2583 (1988), and through GenBank. The teachings of these references are hereby incorporated by reference.

Table 1

DR2 spans positions 1592–1602.

		1571		1592	1602			1632		
1) HPBADW	5'	GCCGGTCCGT	GTGCACTTCG	CTTCACCTCT	CCACGTTGCA	TGGCGACCAC	CGTGAACGCC	CA	3'	(SEQ ID NO: 3)
2) HBVADW2		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
3) HUMPRECX		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
4) HVHEPB		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
5) HBVXCPS		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
6) S50225		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
7) HBVAYWCI		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
8) HPBADWZCG		::::::::::	::::::::::	::::::::::	::::::::::	:::A::::::	::::::::::	::		(SEQ ID NO: 4)
9) HPBADW1		:::::A::::	::::::::::	::::::::::	::::::C:::	:::A::::::	::::::::::	::		(SEQ ID NO: 5)

DEFINITION*

1) HPBADW	Hepatitis B Virus (Subtype ADW)
2) HBVADW2	Hepatitis b virus genome (serotype adw)
3) HUMPRECX	Homo sapiens preC gene
4) HVHEPB	Hepatitis B Virus (HBV 991)
5) HBVXCPS	Hepatitis B Virus X, C, P and S
6) S50225	(HBV variant) [hepatitis B virus [HBV]]
7) HBVAYWCI	Hepatitis B virus (ayw, patient CI)
8) HPBADWZCG	Hepatitis B virus (subtype adw)
9) HPBADW1	Hepatitis B Virus (HBV)

*Sequences according to GenBank. All are human viruses.

It has been determined that the antiviral antisense oligomers of the invention, which are complementary to a region of HBV plus strand DNA comprising DR2; at least 3 and preferably at least 4 conserved 5′ flanking nucleotides; and 0-30, preferably 0-20, more preferably 0-10, and most preferably 4-6 3′ flanking nucleotides, can totally block HBV replication. "Antisense" is a term that means complementary to the sense (or plus) strand. An antisense oligonucleotide interacts in a sequence-specific manner with a cellular nucleic acid target containing a sequence complementary to the antisense molecule. The oligonucleotides of the invention are complementary to the DR2 region of HBV plus strand DNA and to the corresponding region of HBV RNA (which is also of plus strand polarity), and thus interact with these regions, thereby inhibiting HBV viral replication. The interaction of such oligonucleotides with their complementary or "receptor" sequences may result from hybridization interactions, or through other mechanisms which are not yet fully understood. The therapeutic applications of antisense oligonucleotides are described, e.g., in the following review articles: Le Doan *et al.*, Antisense Oligonucleotides as Potential Antiviral and Anticancer Agents, *Bull. Cancer 76*:849-852 (1989); Dolnick, BJ, Antisense Agents in Pharmacology, *Biochem. Pharmacol. 40*:671-675 (1990); and Crooke, *Annu. Rev. Pharmacol. Toxicol. 32*:329-76 (1992).

The invention relates to a composition of matter consisting essentially of an antiviral oligonucleotide, preferably an oligodeoxynucleotide, having a nucleotide sequence substantially complementary to a portion of the plus strand of an HBV genome comprising DR2 plus certain 5′ and, optionally, 3′ flanking sequences, preferably both 5′ and 3′ flanking sequences. Such oligonucleotides will also be

complementary to the corresponding RNAs (e.g, messenger RNA or genomic RNA), which are of plus strand polarity. Because the antisense oligonucleotides are substantially complementary to the DR2 region of HBV, they are capable of hybridizing to the HBV plus strand under physiological conditions. Antisense oligonucleotides of the invention have been shown to be capable of completely inhibiting HBV replication. Accordingly, the invention also relates to methods of inhibiting HBV replication in cells containing HBV, including methods of preventing HBV infection in an animal exposed to HBV, and methods of treating an animal infected with HBV; such animals include, for example, humans and other primates, such as chimpanzees. The invention also relates to pharmaceutical compositions for use in preventing HBV infection in an animal exposed to HBV, or treating an animal infected with HBV. Preferred are such pharmaceutical compositions formulated for parenteral administration. Such pharmaceutical compositions will contain an effective antiviral amount of an oligonucleotide of the invention and a pharmaceutically acceptable carrier.

The invention also relates to a composition of matter consisting essentially of at least one antiviral antisense oligonucleotide substantially complementary to a portion of the plus strand of an HBV genome comprising DR2 plus 5′ and/or 3′ flanking sequences, preferably including residues complementary to all or substantially all of nucleotides 1588 through 1606, preferably all or substantially all of nucleotides 1588 through 1608, of HBV subtype ADW (HPBVADW), or corresponding nucleotides of other HBV strains. Corresponding sequences for strains of HBV other than HBV subtype ADW, e.g., the strains listed in Table 1, *infra*, as well as other strains of HBV that have been and will in the future be

isolated and sequenced, can be determined by those of ordinary skill in the art by aligning sequences for homology, e.g., by using an available database such as GenBank.

Antiviral oligonucleotides of the invention can be supplied to a target cell either exogenously as DNA or RNA, or endogenously, by supplying a DNA sequence from which the desired oligonucleotide may be transcribed by the target cell. In the latter case, the DNA to be expressed may be supplied to the target cell, preferably a hepatocyte, as a recombinant nucleic acid (e.g., a DNA molecule) comprising a DR2 sequence and flanking oligonucleotides, wherein expression of said DNA is capable of inhibiting viral replication. This nucleic acid molecule is characterized in that it (a) is capable of being replicated in a hepatocyte under conditions that normally prevail in the hepatocyte, and (b) is transcribed in a hepatocyte to produce an oligonucleotide substantially complementary to a portion of the plus strand of a hepatitis B viral genome consisting of DR2 and 5′ and 3′ flanking sequences. The sequence transcribed into the antiviral oligonucleotide is preferably operably linked to a cell-specific promoter to direct expression in the hepatocyte. The invention also includes a method for inhibiting hepatitis B virus replication in a cell by introducing the oligonucleotide molecule itself directly into the cell, or by introducing into the cell a nucleic acid which is transcribed within the cell to produce multiple copies of the antiviral oligonucleotide as an oligoribonucleotide.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and oligonucleotide compositions are provided for the inhibition of viral replication.

The antisense oligonucleotide itself may be provided exogenously to a host cell infected with the virus or susceptible to viral infection. Another approach, however, is to provide for expression of the antiviral oligonucleotide in the host cell. In such a method, a DNA transcribable into the antisense oligonucleotide of the invention is incorporated into an expression vector downstream from, and operatively linked to, a suitable promoter which provides for tissue specific or general expression. To treat or prevent viral hepatitis, the DNA of the invention may be placed downstream from a liver specific promoter, in order to induce expression by hepatocytes in the liver; however, it may not be necessary to use a liver-specific promoter, since expression of the antisense oligonucleotides in non-liver cells should be harmless to the cells. The DNA with the appropriate regulatory regions is provided in proper orientation to allow for expression. Methods for constructing such expression vectors are known in the art. See in particular, *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, NY (1989).

A wide variety of transcriptional regulatory sequences may be employed. The signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed.

The expression of the HBV DNA in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of antisense RNA synthesis. Typical promoters include the promoter of the

mouse metallothionine I gene (Hammer, D. *et al.*, *J. Mol. Appl. Gen.* *1*:273-288 (1982)); the Tk promoter of herpes virus (McKnight, S., *Cell* *31*:355-365 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature* *290*:304-310 (1981)); and the like. Other useful promoters include liver specific promoters such as albumin, alpha-fetoprotein, alpha-1-antitrypsin, retinol-binding protein, asialoglycoprotein receptor, and viral promoters and enhancers such as those of cytomegalovirus; herpes simplex I and II viruses; hepatitis A, B, and C viruses, and Rous sarcoma virus (RSV) (Fang, X.J. *et al.*, *Hepatology* *10*:781-787 (1989)). Such liver-specific promoters are expected to be particularly useful when a DNA sequence of the invention is placed in a vector which is capable of transforming hepatocytes. The gene is placed downstream from a suitable promoter which provides for tissue specific or general expression. The DNA will be transcribed to produce RNA which will hybridize with its target RNA, thereby inhibiting viral replication. One particular vector useful for this task would be one based on an adenoviral system, such as described by Morsey *et al.*, Abstract SZ 109, "Efficient Adenoviral Gene Transduction in Human and Mouse Hepatocytes *In Vitro* and in Mouse Liver *In Vivo*", in *J. of Cellular Biochemistry*, Suplement 17E, Keystone Symposia on Molecular and Cellular Biology, March 29-April 25, 1993, or alternatively, a retroviral vector such as LNL6, a derivative of the Moloney murine leukemia virus, as described in Rosenberg *et al.*, *N. Eng. J. Med.* 323, No. 9:570-578 (1990). Gene transfer into hepatocytes using a defective Herpes Simplex viral vector is described by Lu *et al.*, Abstract, page 66, Abstracts of the Meeting on Gene Therapy, Sept. 22-26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. This latter approach is especially valuable for introducing genes

into non-dividing hepatocytes, since HSV-1 does not require genomic integration for expression. The DNA and the requisite regulatory elements may also be introduced into hepatocytes using an asialoglycoprotein carrier system as described in Wu *et al.*, *Biotherapy* 3:87-95 (1991).

The desired viral DNA and operably linked promoter may be introduced into a recipient cell either as a non-replicating DNA or RNA molecule, which may be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the desired receptor molecule occurs through the transient expression of the introduced sequence. Where more long-term expression is desired, the sequence may be integrated into the host chromosome. Alternatively, the introduced sequence may be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host; such vectors include the cDNA expression vectors described by Okayama, H., *Mol. Cell. Bio.* 3:280 (1983), and others. Viral vectors include retrovirus vectors as taught in W089/07136 (specifically for expression in hepatocytes) and the references cited therein.

In the methods of the invention, either the antisense oligonucleotide itself, or a DNA which is transcribable into the antisense RNA of the invention, is introduced into the cells of an animal suffering from the viral disease.

As used herein, "substantially complementary" means that an antisense oligonucleotide of the invention is capable of hybridizing with its RNA or DNA target under physiological conditions. HBV nucleotide sequence numbering herein is made with reference to the numbering of Hepatitis B Virus (Subtype ADW), according to GenBank. Corresponding sequences for HBV strains and subtypes,

other than HBV subtype ADW, e.g., the strains listed in Table 1, *infra*, as well as other strains of HBV that have been and will in the future be isolated and sequenced, can be determined by those of ordinary skill in the art by aligning sequences for homology using an available database such as GenBank. This allows for selection of sequences specific for non-human mammalian species or for human HBV strains prevalent in particular populations or geographic areas. Alternatively, corresponding consensus or conserved sequences having broader applicability may be readily determined by comparing corresponding sequences from multiple HBV strains. It is contemplated that such corresponding sequences are functional equivalents of the sequence for ADW and ADW1.

The methods and oligonucleotide compositions of the present invention can be utilized to prevent viral infection as well as to treat viral infections. The compositions comprising vectors containing nucleic acids transcribable into the anti-HBV oligonucleotides of the invention may be administered to prevent a virus infection or to combat the virus once it has entered the host.

As used herein, "consisting essentially of" has its usual meaning, i.e., that one or more compositions of matter of the invention may be used together, either in admixture or combined in a single molecule, with other materials that do not alter the essential nature of the invention. For example, while the antisense oligonucleotide sequences of the invention are essential to the invention, it is contemplated that they may be used in admixture or in chemical combination with one or more other materials, including other oligonucleotides antisense to other portions of HBV RNA; materials that increase the biological stability of the oligonucleotides; or materials that increase their

ability to penetrate selectively their hepatocyte target cells, and reach and hybridize their target RNA. Furthermore, it is recognized that oligonucleotides may be modified to achieve greater stability, including backbone modifications such as phosphorothioates, methylphosphonates, phosphorodithioates, phosphoroamidates, phosphate esters, and other modifications as described in Uhlman and Peyman, Antisense Oligonucleotides: A New Therapeutic Principle. Chemical Reviews 90(4):544-584 (1990), at 546-560, the teaching of which is hereby incorporated by reference. All such modifications are contemplated equivalents of the antisense oligonucleotides of the invention.

The following discussion provides examples of the kinds of modifications that may be employed, but those of skill in the art will readily recognize others. For example, the antisense oligonucleotides may be provided in stabilized form, e.g., with phosphotriester linkages, or by blocking against exonuclease attack with methylphosphonodiester linkages, with 3' deoxythymidine, as a phenylisourea derivative, or by linking other molecules such as aminoacridine or polylysine to the 3' end of the oligonucleotide. See e.g., Anticancer Research 10:1169-1182, at 1171-2 (1990), the teaching of which is incorporated herein by reference. For antisense oligonucleotides supplied exogenously, increased selectivity for hepatocytes may be achieved by linking antisense oligonucleotides of the invention to natural ligands such as ASOR (asialoorosomucoid) or to synthetic ligands that will bind to the hepatic asialoglycoprotein (ASGP) receptor. See e.g., Biochemistry 29, No. 43 (1990), Spiess, "The Asialoglycoprotein Receptor: A Model for Endocytic Transport Receptors". See also Wu and Wu, *J. Biol.Chem. 267*, No. 18:12436-12439 (1992), reporting inhibition of HBV viral gene expression and replication

in HepG2 cells by a 21-mer oligonucleotide complementary to the HBV polyadenylation signal. The oligomer was complexed to a (poly)L-lysine-asialoorosomucoid conjugate that targets the asialoglycoprotein receptor of hepatocytes. In another embodiment, ribozymes may be targeted by linking to an oligonucleotide of the invention, since there are a number of ribozyme target cleavage sites in the DR2 region of the viral RNA. See e.g., Von Weiszaecker F, Blum HE, Wands JR, Three ribozymes transcribed from a single DNA template efficiently cleave hepatitis B virus pregenomic RNA, *Biochem. Biophys. Res. Commun. 189*:743-748 (1992). The teaching of the foregoing references is incorporated herein by reference.

In general, a high efficiency, cell-specific delivery system for *in vivo* therapeutic use may utilize a number of approaches, including the following: 1) specific delivery through hepatocyte specific receptor mediated process such as the asialoglycoprotein receptor, as discussed above; 2) delivery of antisense oligodeoxynucleotides in liposomes with or without specific targeting with monoclonal antibodies directed against specific cell surface receptors; 3) retrovirus-mediated transfer of DNA expressing the antisense construct of interest; and 4) direct targeting to cells of antisense oligodeoxynucleotides following conjugation to monoclonal antibodies that are specific for cell surface receptors that function in a receptor-mediated endocytotic process; 5) specific delivery to hepatocytes via a replication-defective HBV vector. To modify hepadnaviral infection *in vivo*, a hepatocyte-specific delivery system whereby substantially all hepatocytes are provided with an effective amount of the antisense construct will probably be required.

The antisense compositions of the invention may be administered as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a pharmaceutically acceptable carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice. The dosage administered will of course vary depending upon known pharmacokinetic/ pharmacodynamic characteristics of the particular agent and its mode and route of administration, as well as the age, weight, and health (including renal and hepatic function) of the recipient, the nature and extent of disease, kind of concurrent therapy, frequency and duration of treatment, and the effect desired. Usually a daily dose of active ingredient can be about 0.1 to 100 mg per kilogram of body weight. Ordinarily 0.5 to 50, and preferably 1 to 10 mg per kg of body weight per day given in divided doses or in sustained release form (including sustained intravenous infusion) will be effective to achieve the desired effects.

Dosage forms suitable for internal administration generally contain about 1 milligram to about 500 milligrams of active ingredient per unit. The active ingredient will ordinarily be present in an amount of about 0.5 to 95% by weight of the total pharmaceutical preparation. It is expected that the antisense oligonucleotide compositions of the invention may be administered parenterally (e.g., intravenously, preferably by intravenous infusion). For parenteral administration, the compositions will be formulated as a sterile, non-pyrogenic solution, suspension, or emulsion. The preparations may be supplied as a liquid formulation or lyophilized powder to be diluted with a pharmaceutically acceptable, sterile, non-pyrogenic parenteral vehicle of suitable tonicity, e.g., water for

injection, normal saline, or a suitable sugar-containing vehicle, e.g., D5W, D5/0.45, D5/0.2, or a vehicle containing mannitol, dextrose, or lactose. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field, and in the USP/NF.

The oligonucleotides or their derivatives may also be administered in liposomes or microspheres (microparticles). Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Pat. No. 4,789,734 describes methods for encapsulating biological materials in liposomes. The material is dissolved in an aqueous solution; the appropriate phospholipids and lipids added, along with surfactants, if required; and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Chapter 14, "Liposomes", *Drug Carriers in Biology and Medicine,* pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the oligomers or their derivatives can be incorporated into microspheres, and implanted for slow release over a period of time. See, e.g., U.S. Patents Nos. 4,925,673 and 3,625,214.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE I

Transfection of HuH 7 cells

HuH 7 hepatoma cells (*Cancer Research 42*:3858-3863 (1982)) were seeded into 6 well plates (35mm/well) and grown to 70-90% confluency in Dulbecco's Minimal

Essential Medium supplemented with 10% fetal bovine serum. Cells were transfected according to a modification of the procedure described by Chen and Okayama, *Mol.Cell. Biol.* 7:2745-2752 (1987). [The modification in this procedure was to incubate the cells in an atmosphere of 5% CO_2 throughout the experiment, as opposed to incubating the cells in a lower CO_2 atmosphere during the actual transfection step.]

Cells were transfected in duplicate with 35 to 50 ng replication-competent HBV plasmid construct containing HBV DNA sequences [head-to-tail dimer of HBV DNA HBsAg subtype adw in pGEM-72f(+) (Promega)], which was obtained from J. Wands, MGH, Boston [see Blum et al., J. Virol. 65(4):1836-1842 (1991)]. Cell supernatants were harvested post-transfection on a daily basis for up to 6 days, and cells refed daily with 2mls of medium/well. The supernatants were stored at 4° C until assayed for the presence of HBsAg using the Auszyme Monoclonal Diagnostic Kit, a commercially available enzyme immunoassay from Abbott Laboratories, North Chicago, IL. HBsAg is a marker for viral replication, and the ability of oligonucleotide to block HBsAg production indicates inhibition of viral replication.

Oligonucleotides

The following oligodeoxynucleotides were synthesized in a Milligen Biosearch 8750 DNA synthesizer, using asialoethyl-phosphoramidite syntheses (*Tetrahedron Lett 22*: 1859-1862 (1981)):

Oligo ID# HBV 10011
Description DR2 antisense
Sequence: 5' CAA CGT GCA GAG GTG AAG CGA 3' (SEQ ID NO: 6)

Oligo ID# HBV 10012
Description DR2 scramble of antisense
Sequence: 5' AGC GAA GTG AGG ACG TGC AAC 3' (SEQ ID NO: 7)

Oligo ID# CP 10053
Description DR1 antisense
Sequence: 5' TTA GGC AGA GGT G AA AAA GTT 3' (SEQ ID NO: 8)

Oligo ID# CP 10052
Description DR1 scramble of antisense
Sequence: 5' ATC GGA GGA TGG TTA AAT GAA 3' (SEQ ID NO: 9)

Purification of Oligodeoxynucleotides.

Antisense and scrambled oligodeoxynucleotides were purified after NH_4OH detachment (55°C, 6 hours) and NAP 25 column (Pharmacia) desalting with 0.1 M $NaHCO_3$ by reverse phase HPLC (trityl on, TEAA 0.1 M, pH 7.25/acetonitrile gradient). The oligodeoxynucleotides were lyophilized, deblocked with 1 M acetic acid for 1 hour, neutralized with 1.0 M $NaHCO_3$, passed through a NAP 10 column, and then lyophilized to dryness.

Results

HuH 7 hepatoma cells transfected with 35-50 ng of plasmid containing HTD HBV subtype adw2 genome, and treated with 8 μg/well of the DR2 antisense oligonucleotide HBV 10011 (SEQ ID NO: 6) at the time of transfection, produced significantly less HBsAg over the six day test period than did control transfected cells not treated with an antisense oligonucleotide. In contrast, the DR1 antisense oligonucleotide CP 10053 (SEQ ID NO: 8) did not cause a detectable decrease in the accumulation of HBsAg, compared to control supernatants from transfected cells untreated with oligonucleotide. The two scrambled antisense oligonucleotides, HBV 10012

(SEQ ID NO: 7) and CP 10052 (SEQ ID NO: 9) slightly decreased the amount of HBsAg present in supernatants. The observed difference in the activity of DR1 and DR2 antisense oligonucleotides is surprising considering that DR1 and DR2 share 11 identical nucleotides and both play a critical role in HBV replication. Furthermore, DR1 appears to be involved at an earlier step in HBV replication, since synthesis of the first DNA strand is believed to be initiated within DR1.

It is thought that the DR2 antisense oligonucleotide may be acting at any of several possible levels. It may bind to and have an antisense effect on the HBV 3.5 kb pregenomic RNA, the HBV 2.1 kb mRNA and/or the HBV 2.4 kb mRNA. The DR2 sequence is present in both the polymerase and the X protein ORFs. The antiviral oligonucleotides might also bind to the oligoribonucleotide primer generated during HBV replication, thereby preventing oligoribonucleotide priming at the DR2 site on minus strand DNA and inhibiting plus strand DNA synthesis. It should be recognized, however, that oligomers of the invention are also complementary to and are capable of hybridizing with plus strand HBV DNA containing DR2. While the nucleotide sequence of this region is highly conserved amoung different HBV strains, a finding which suggests that nucleotide sequence and its function in viral replication may be important, secondary and tertiary structures may also be important with respect to interaction with protein. The invention as claimed, however, is not predicated on any particular mechanism or mechanisms through which the DR2 oligonucleotides act to block HBV replication.

DR1 and DR2 share 11 identical nucleotides, but have different flanking sequences. Flanking regions of both DR1 and DR2 are also conserved among various species

and strains of HBV, indicating the importance of these regions in HBV DNA replication. Moreover, flanking sequences of DR2 of many human and woodchuck HBVs contain a two-fold symmetry, making the majority of DR2 sequence and the 5'-flanking region of DR2 into a single-stranded loop. A putative secondary structure of the DR2 region of HBV DNA is shown in Fig. 2, using a suboptimal RNA folding program (*see* Jaeger, JA, Turner, DH, and Zuker, M, *Proc. Natl. Acad. Sci. USA* 86:7706 (1989)). However, secondary structure evaluation of the two DR1 sequences in the pregenomic RNA suggested that one of the DR1 sequences might also be found as a single stranded loop. Nevertheless, as described above, DR1 antisense did not decrease HBsAg accumulation.

The proposed mechanism of action of antisense oligonucleotides requires hybridization of an oligonucleotide to its complementary sequence in the RNA target. Therefore, for an antisense oligonucleotide to be effective, the complementary target sequence must be available for hybridization. In most cases, target mRNA is not single-stranded random coil, but rather contains secondary and tertiary structures. Target RNA structure has been shown to affect the affinity and rate of oligonucleotide hybridization, as well as the efficacy of antisense oligonucleotides [*See* Yoon, K. Turner, D.H., and Tinco, I, Jr., *J. Mol. Biol. 99*:507 (1975); Freier, S.M. and Tinoco, I., Jr., *Biochemistry 14*:3310 (1975); Uhlenbeck, O.C., *J. Molec. Biol 65*:25 (1972); Herschlag D. and Cech, T.R., *Biochemistry 29*:10159 (1990); and Fedor, M. J. and Uhlenbeck. O.C., *Proc. Natl. Acad. Sci. U.S.A. 87*:1668 (1990)]. Thus, when designing antisense oligonucleotides it may be helpful to consider mRNA structure and the potential influence of this structure on oligonucleotide hybridization. For example, it was recently reported that the tightest binding of antisense

oligonucleotides occurs at target sites for which disruption of the target stucture was minimal (*see* Lima, W.F., Monia, R.P., Ecker, D.J. and Freier, S.M. *Biochemistry 31*:12055 (1992)). Thus, consideration of the target secondary structure suggests that single-stranded regions should be selected over double-stranded regions. However, not all single-stranded regions exhibit structures favorable to hybridization (e.g., the single-stranded portions of loops). It was reported that oligonucleotides complementary to the 5′ side of the single-stranded loop of kras RNA may exhibit tighter binding affinity compared to the oligonucleotides complementary to the 3′ side of the loop (Lima *et al.*, *supra*). However, tat and tar regions of HIV mRNA, which also contain a single-stranded loop, did not exhibit any preferential binding to the 3′ side of the loop [Ecker et al., *Science 257*:958-961 (1992)].

Based on the current state of knowledge, target sites for antisense attack must still be determined experimentally, although the presence of a single-stranded loop in a region of mRNA may indicate to a logical point for research. However, studies of putative RNA secondary structure may provide insight into the results that have been obtained experimentally.

The oligonucleotides were tested for nonspecific cytotoxicity by means of the MTT assay. The assay is dependent on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) by the mitochondrial hydrogenase of viable cells to form a blue formazan product (D. Gerlier et al., J. Immunol. Methods 94:57-63 (1986); D.S. Heo et al., Cancer Res 50:3681-3690 (1990)). This assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells in culture. HuH 7 cells incubated in the presence of an

oligonucleotide of the invention or a scrambed control exhibited a reading in the MTT assay similar to that observed with HuH 7 cells incubated without addition of oligonucleotide. Thus, the decrease in HBsAg expression observed with the oligonucleotides of the invention appears to be a specific inhibition, not a general cytotoxic effect of the oligonucleotides.

EXAMPLE 2

In order to elucidate further the structure-activity relationships of antisense oligonucleotides complementary to plus strand HBV DNA in the DR2 region, a number of additional olignucleotides were synthesized and tested for antiviral activity in the manner described above.

Oligonucleotides

The following oligodeoxynucleotides were synthesized in a Milligen Biosearch 8750 DNA synthesizer, using asialoethyl-phosphoramidite syntheses (*Tetrahedron Lett 22*: 1859-1862 (1981)):

Oligo ID# CJP 114
Description DR2 antisense
Sequence: 5′ CGA CGT GCA GAG GTG AAG CGA 3′ (SEQ ID NO: 10)

Oligo ID# CJP 113
Description DR2 antisense
Sequence: 5′ TGC AGA GGT GAA GC 3′ (SEQ ID NO: 11)

Oligo ID# CJP 112
Description DR2 antisense
Sequence: 5′ TGA AGC GAA GTG CA 3′ (SEQ ID NO: 12)

Oligo ID# CJP 111
Description DR2 antisense
Sequence: 5′ GAG GTG AAG CGA AG 3′ (SEQ ID NO: 13)

Oligo ID# CJP 110
Description DR2 antisense
Sequence: 5′ GGT GAA GCG A 3′ (SEQ ID NO: 14)

Oligo ID# CJP 109
Description DR2 antisense
Sequence: 5′ CGT GCA GAG GTGAAG CGA AGT 3′ (SEQ ID NO: 15)

Oligo ID# CJP 108
Description DR2 antisense
Sequence: 5′ CGA CGT GCA GAG GTG AAG CGA AGT 3′ (SEQ ID NO: 16)

Oligo ID# CJP 101
Description DR2 antisense
Sequence: 5′ GGT GAA GCG A 3′ (SEQ ID NO: 17)

Oligo ID# CJP 100
Description DR2 antisense
Sequence: 5′ CGA CGT GCA GA 3′ (SEQ ID NO: 18)

RESULTS

Since DR2 antisense oligodeoxynucleotide HBV 10011 (5′ CAA CGT GCA GAG GTG AAG CGA 3′; SEQ ID NO: 6) exhibited strong anti-HBV activity, a series of other oligodeoxynucleotides complementary to the DR2 region of plus strand HBV DNA were synthesized and tested in the manner described above. Originally, a series of DR2 antisense oligonucleotides was designed based on the putative secondary structure of the DR2 loop region (Figure

1), with the rationale being to target antisense oligonucleotides to the putative single-stranded region of the DR2 loop, which may be favorable for hybridization in a manner similar to H-ras mRNA (Lima, 1992). The antiviral effect of each of these oligonucleotides is shown in Table 2, and discussed below.

HBV 10011 is a 21-mer consisting of nucleotides complementary to the entire eleven-nucleotide DR2 as well as four 5′ flanking nucleotides and six 3′ flanking nucleotides. The oligodeoxynucleotide designated CJP 114 is a 21-mer differing from HBV 10011 only with respect to a single nucleotide at n.t.n. 1607 (numbering relative to HPBV ADW from GenBank). Comparing the DR2 region of various HBV strains using GenBank revealed a nucleotide change from T in HBV ADW to C in HBV ADW1 and HBV ADRCG. CJP 114, which contained a G rather than an A at n.t.n. 1607, is complementary to the plus stand of the HBV ADW1 strain. Although the assay for inhibition of HBV replication employed in these experiments used an HBV ADW2 strain, which has a T at n.t.n. 1607, CJP 114 nevertheless blocked replication of HBV ADW2.

Oligodeoxynucleotide CJP 108 is a 24-mer, differing from CJP 114 in that it has an additional three nucleotides complementary to the DR2 5′ flanking region. Surprisingly, CJP 108 does not inhibit HBV replication. Similarly, CJP 109, a 21-mer lacking (relative to CJP 108) three nucleotides from the DR2 3′ flanking region, does not inhibit HBV replication. Oligonucleotide CJP 126, having the sequence of HBV 10011 with a blocking group at the 3′ end, also exhibited decreased antiviral activity. This provides further evidence that flanking sequences or groups at the 3′-end of the HBV 10011 sequence may be detrimental to antiviral activity.

It appears that nucleotides complementary to the entire DR2 region as well as several nucleotides from both the 5′ and 3′ flanking regions are required for antiviral activity. Oligdeoxynucleotide CJP 113, a 14-mer containing nucleotides complementary to the entire DR2 but only two nucleotides from the 5′ flanking region and a single nucleotide from the 3′ flanking region, did not block HBV replication. CJP 111, containing nucleotides complementary to eight of eleven of the DR2 nucleotides and six nucleotides from the 5′ flanking region, did not inhibit HBV replication. CJP 101 and CJP 110, both 10-mers complementary to six of the eleven DR2 nucleotides and five 5′ flanking nucleotides, did not inhibit HBV replication. CJP 112, a 14-mer containing nucleotides complementary to four of eleven of the DR2 nucleotides plus ten nucleotides from the 5′ flanking region, did not inhibit HBV replication. CJP 100, an 11-mer complementary to five of the eleven DR2 nucleotides and six 3′ flanking nucleotides, did not inhibit HBV replication.

Surprisingly, antisense oligonucleotides designed to hybridize to a putative loop region of the DR2 region (CJP 111 and CJP 110) and antisense oligonucleotides designed to hybridize to a putative loop and either 5′ or 3′ flanking region (CJP 113, CJP 109, CJP 108, CJP 112) did not exhibit antiviral activity. However, HBV 10011 and CJP 114, which contain a single nucleotide difference, did exhibit antiviral activity. This suggests that two nucleotides at the 5′ end of antisense oligonucleotides HVB 10011 and CJP 114 may not be important for antiviral activity. To test this hypothesis, oligonucleotide CJP 140, identical to HBV 10011 and CJP 114 except that the two 5′ nucleotides of the latter were deleted, was synthesized and tested. CJP 140 was found to inhibit completely HVB replication.

Accordingly, it appears that more than four 5′ flanking nucleotides are unnecessary for full antiviral activity. While up to twelve, preferably only up to six, 5′ flanking nucleotides may be included, oligomers having only up to four 5′ flanking nucleotides are more preferred for ease of synthesis and pharmaceutical delivery.

To further elucidate these structure-activity relationships, oligonucleotide HBV 1018 (SEQ ID NO: 19), which is an 11-mer complementary to the DR2 sequence alone, without either 3′ or 5′ flanking sequences, was synthesized and tested. It failed to inhibit HBV replication.

Oligonucleotide CJP 150 (SEQ ID NO: 20), which is identical to CJP 114 except for a single nucleotide deletion at the 3′ end, reduced the amount of HBsAg in transfected cell supernatants to 5% of levels found in supernatants of cells transfected with the HBV plasmid DNA alone. Since oligonucleotide CJP 150 exhibits a slightly decreased antiviral activity relative to oligonucleotides HBV 10011 and CJP 114, it appears that the A residue complementary to nucleotide 1588 of the HBV genome may be necessary for full antiviral activity. A modified oligonucleotide having the sequence 5′-ACGTGCAGAGGTGAAGCG-3′ (SEQ ID NO: 21) would also be expected to have antiviral activity.

Thus, only oligonucleotides targeted to a narrow region of DR2 sequence exhibit antiviral activity. These experimental results appear in Table 2 below. There appears to be no apparent correlation between the putative secondary structure (Fig. 1) and antiviral activity observed experimentally. However, not only RNA structure but also its interaction with proteins may be important for antiviral activity. This must be determined experimentally.

Preliminary evidence indicates that the oligonucleotides of the invention exhibit specific antiviral activity against HBV since these oligonucleotides are non-toxic in a mitochondrial enzyme assay and there is no evidence, by gross morphology, of toxicity to hepatocytes in culture.

Table 2

		DR2 (1592–1602); positions: 1582, 1592, 1602, 1613	
HPBVADW (sense)		5' TGCACTTCGC TTCACCTCTGC ACGTTGCATGG 3'	(SEQ ID NO: 22)
HPBADW1 (sense)		:::::::::: ::::::::::: ::::C::::::	(SEQ ID NO: 23)
ANTISENSE OLIGONUCLEOTIDE (Shown 3'-5'):			
HBV 10011	(+)	AGCG AAGTGGAGACG TGCAAC	(SEQ ID NO: 6)
CJP 114	(+)	AGCG AAGTGGAGACG TGCAGC	(SEQ ID NO: 10)
CJP 140	(+)	AGCG AAGTGGAGACG TGCA	(SEQ ID NO: 24)
CJP 150	(+)	GCG AAGTGGAGACG TGCAGC	(SEQ ID NO: 20)
HBV 1018	(-)	AAGTGGAGACG	(SEQ ID NO: 19)
CJP 113	(-)	CG AAGTGGAGACG T	(SEQ ID NO: 11)
CJP 109	(-)	TGAAGCG AAGTGGAGACG TGC	(SEQ ID NO: 15)
CJP 108	(-)	TGAAGCG AAGTGGAGACG TGCAGC	(SEQ ID NO: 16)
CJP 112	(-)	ACGTGAAGCG AAGT	(SEQ ID NO: 12)
CJP 111	(-)	GAAGCG AAGTGGAG	(SEQ ID NO: 13)
CJP 101	(-)	AGCG AAGTGG	(SEQ ID NO: 17)
CJP 100	(-)	AGACG TGCAGC	(SEQ ID NO: 18)

(+) indicates positive antiviral activity
(-) indicates lack of antiviral activity

The experimental results presented in Table 2 indicate that a region comprised of DR2 plus flanking sequences is important for the normal function of HBV. Oligonucleotides HBV 10011 and CJP 114, both of which map to nucleotide positions 1588 to 1609 inclusive of the hepatitis B genome (numbering relative to HPBV ADW from GenBank), prevent the accumulation of detectable levels of HBsAg in cell supernatant. HBV 10011 and CJP 114 differ from each other only at position 1607, with HBV 10011 containing an A and CJP 114 containing a G. This suggests that basepairing between the antiviral oligonucleotide and the viral nucleic acid at position 1607 may not be necessary for the antiviral effect seen experimentally. Accordingly, an oligonucleotide with the sequence 3′ AGCGAAGTGGAGACGTGCA 5′ (OLIGO CJP 140; SEQ ID NO: 24), was synthesized and found to have an antiviral effect.

Antiviral oligonucleotides CJP 101, which corresponds to positions 1588 to 1598, does not inhibit HBV surface antigen expression. This indicates that some or all of the nucleotides mapping from positions 1599 to 1609 are essential for an antiviral effect. Oligonucleotide CJP 100, which corresponds to position 1599 to 1609, has no effect on the accumulation of Hepatitis B surface antigen. This indicates that some or all of the nucleotides corresponding to positions 1588 to 1597 are also critically important for the inhibition effected by oligonucleotides HBV 10011 and CJP 114. Oligonucleotide CJP 113 corresponds to positions 1590 to 1603 and is not active as an inhibitor of Hepatitis B replication. This indicates that all or some of the nucleotides mapping at positions 1588, 1589, 1604, 1605, 1606, 1607, 1608, and 1609 are required for antiviral activity. Oligonucleotide CJP 108 is identical to CJP 114, except that CJP 108 contains an additional three

nucleotides at the 3′ end; however, CJP 108 does not exhibit antiviral activity. One or more of the extra three 3′ nucleotides appear to abolish the antiviral activity. Oligonucleotide CJP 109 also includes the same three 3′ terminal nucleotides as does CJP 108, and CJP 109 is also inactive in the assay. Similarly, oligonucleotides CJP 112 and CJP 111 exhibit no antiviral activity in the assay. Both contain a 3′ nucleotide or nucleotides which may abolish activity, as well as lacking important 5′ sequences present in HBV 10011 and CJP 114.

Consistent with the foregoing, the following DR2 oligonucleotides (shown 5′ to 3′) would also be expected to exhibit anti-HBV activity:

OLIGO CJP 141: CGTGCAGAGGTGAAGCGA (SEQ ID NO: 25);
OLIGO CJP 142: GTGCAGAGGTGAAGCGA (SEQ ID NO: 26);
OLIGO CJP 143: TGCAGAGGTAAGCGA (SEQ ID NO: 27);
OLIGO CJP 144: GCAGAGGTGAAGCGA (SEQ ID NO: 28);
OLIGO CJP 145: CAGAGGTGAAGCGA (SEQ ID NO: 29);
OLIGO CJP 146: AGAGGTGAAGCGA (SEQ ID NO: 30);
OLIGO CJP 147: GAGGTGAAGCGA (SEQ ID NO: 31);
OLIGO CJP 148: AGGTGAAGCGA (SEQ ID NO: 32);
OLIGO CJP 149: CGACGTGCAGAGGTGAAGC (SEQ ID NO: 33);
OLIGO CJP 150: CGACGTGCAGAGGTGAAGCG (SEQ ID NO: 20);
OLIGO CJP 151: CGACGTGCAGAGGTGAAG (SEQ ID NO: 34);
OLIGO CJP 152: CGACGTGCAGAGGTGAA (SEQ ID NO: 35);
OLIGO CJP 153; CGACGTGCAGAGGTGA (SEQ ID NO: 36);
OLIGO CJP 154: CGACGTGCAGAGGTG (SEQ ID NO: 37);
OLIGO CJP 155: CGACGTGCAGAGGT (SEQ ID NO: 38);
OLIGO CJP 156: CGACGTGCAGAGG (SEQ ID NO: 39);
OLIGO CJP 157: CGACGTGCAGAG (SEQ ID NO: 40);
OLIGO CJP 158: AACGTGCAGAGGTGAAGCGA (SEQ ID NO: 41);
OLIGO CJP 159: CGACGTGCAGAGGTGAAGCGAAG (SEQ ID NO: 42);
OLIGO CJP 160: CGACGTGCAGAGGTGAAGCGAA (SEQ ID NO: 43).

Additional active sequences may be determined based on the results of experiments carried out to delineate the precise 3′ and 5′ boundaries of activity, and then selecting common nucleotide sequences.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are incorporated herein by reference to the same extent as if each individual publication or patent application were specifically and individually stated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those of skill in the art that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Coney, Leslie R.
Pachuk, Catherine J.
Yoon, Kyonggeun

(ii) TITLE OF INVENTION: ANTI-HEPATITIS B VIRAL OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 52

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/172,538
(B) FILING DATE: 23 December 1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Fraser, Janis K.
(B) REGISTRATION NUMBER: 34,819
(C) REFERENCE/DOCKET NUMBER: 06120/002WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TATAAA 6

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	11
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTCACCTCTG C 11

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	62
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCGGTCCGT GTGCACTTCG CTTCACCTCT GCACGTTGCA TGGCGACCAC CGTGAACGCC 60

CA 62

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	62
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCGGTCCGT GTGCACTTCG CTTCACCTCT GCACGTTGCA TGGAGACCAC CGTGAACGCC 60

CA 62

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	62
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCCGGACCGT GTGCACTTCG CTTCACCTCT GCACGTCGCA TGGAGACCAC CGTGAACGCC 60

CA 62

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	21
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CAACGTGCAG AGGTGAAGCG A 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	21
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGCGAAGTGA GGACGTGCAA C 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	21
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTAGGCAGAG GTGAAAAAGT T 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	21
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATCGGAGGAT GGTTAAATGA A 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	21
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGACGTGCAG AGGTGAAGCG A 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TGCAGAGGTG AAGC 14

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGAAGCGAAG TGCA 14

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GAGGTGAAGC GAAG 14

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGTGAAGCGA 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGTGCAGAGG TGAAGCGAAG T 21

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGACGTGCAG AGGTGAAGCG AAGT 24

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGTGAAGCGA 10

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	11
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGACGTGCAG A 11

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	11
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AAGTGGAGAC G 11

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCGAAGTGGA GACGTGCAGC 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	18
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ACGTGCAGAG GTGAAGCG 18

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TGCACTTCGC TTCACCTCTG CACGTTGCAT GG 32

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TGCACTTCGC TTCACCTCTG CACGTCGCAT GG 32

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

ACGTGCAGAG GTGAAGCGA 19

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGTGCAGAGG TGAAGCGA 18

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTGCAGAGGT GAAGCGA 17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	15
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TGCAGAGGTA AGCGA 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	15
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCAGAGGTGA AGCGA 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CAGAGGTGAA GCGA 14

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	13
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

AGAGGTGAAG CGA 13

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	12
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GAGGTGAAGC GA 12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	11
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

AGGTGAAGCG A 11

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	19
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

CGACGTGCAG AGGTGAAGC 19

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	18
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CGACGTGCAG AGGTGAAG 18

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	17
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CGACGTGCAG AGGTGAA 17

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	16
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CGACGTGCAG AGGTGA 16

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	15
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CGACGTGCAG AGGTG 15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	14
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CGACGTGCAG AGGT 14

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

CGACGTGCAG AGG 13

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

CGACGTGCAG AG 12

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

AACGTGCAGA GGTGAAGCGA 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

CGACGTGCAG AGGTGAAGCG AAG 23

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

CGACGTGCAG AGGTGAAGCG AA 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	201
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

TCTGCCGTAC CGTCCGACCA CGGGGCGCAC CTCTCTTTAC GCGGACTCCC CGTCTGTGCC 60

TTCTCATCTG CCGGACCGTG TGCACTTCGC TTCACCTCTG CACGTCGCAT GGAGACCACC 120

GTGAACGCCC ACGGAACCCT GCCCAAGGTC TTGCATAAGA GGACTCTTGG ACTTTCAGCA 180

ATGTCAACGA CCGACCTTGA G 201

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	47
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TGGGCGTTCA CGGTGGTCGC CATGCAACGT GCAGAGGTGA AGCGAAG 47

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	47
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

TGGGCGTTCA CGGTGGTCTC CATGCAACGT GCAGAGGTGA AGCGAAG 47

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	47
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

TGGGCGTTCA CGGTGGTCGC CATGCGACGT GCAGAGGTGA AGCGAAG 47

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	47
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

TGGGCGTTCA CGGTGGTCTC CATGCGACGT GCAGAGGTGA AGCGAAG 47

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

GACGTGCAGA GGTGAAGCGA 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

AACGTGCAGA GGTGAAGCGA 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	19
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

GACGTGCAGA GGTGAAGCG 19

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	19
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

AACGTGCAGA GGTGAAGCG 19

CLAIMS

1. An oligonucleotide having antiviral activity against hepatitis B virus (HBV), consisting essentially of a sequence substantially complementary to a portion of plus (+) strand of the HBV genome, said portion consisting of the DR2 11-mer (SEQ ID NO: 44) plus 0-6 nucleotides of 5′ flanking sequence and 0-30 nucleotides of 3′ flanking sequence.

2. An oligonucleotide of Claim 1, wherein said oligonucleotide has the sequence:
5′-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 45),
5′-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 46),
5′-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 47), or
5′-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3′
(SEQ ID NO: 48),
or a portion thereof, provided that when said oligonucleotide is a ribonucleotide, "T" in each of said sequences represents "U".

3. An oligonucleotide of Claim 2, wherein said oligonucleotide is at least 15 nucleotides in length.

4. An oligonucleotide of Claim 3, wherein said oligonucleotide comprises the sequence
5′-ACGTGCAGAGGTGAAGCG-3′ (SEQ ID NO: 21).

5. An oligonucleotide of Claim 1, having one of the following sequences:
5′-CAACGTGCAGAGGTGAAGCGA-3′ (OLIGO HBV 10011; SEQ ID NO: 6);

5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);
5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);
5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);
5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);
5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);
5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51);
5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52);
5'-CGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 141: SEQ ID NO: 25);
5'-GTGCAGAGGTGAAGCGA-3' (OLIGO CJP 142: SEQ ID NO: 26);
5'-TGCAGAGGTAAGCGA-3' (OLIGO CJP 143: SEQ ID NO: 27);
5'-GCAGAGGTGAAGCGA-3' (OLIGO CJP 144: SEQ ID NO: 28);
5'-CAGAGGTGAAGCGA-3' (OLIGO CJP 145: SEQ ID NO: 29);
5'-AGAGGTGAAGCGA-3' (OLIGO CJP 146: SEQ ID NO: 30);
5'-GAGGTGAAGCGA-3' (OLIGO CJP 147: SEQ ID NO: 31);
5'-AGGTGAAGCGA-3' (OLIGO CJP 148: SEQ ID NO: 32);
5'-CGACGTGCAGAGGTGAAGC-3' (OLIGO CJP 149: SEQ ID NO: 33);
5'-CGACGTGCAGAGGTGAAG-3' (OLIGO CJP 151: SEQ ID NO: 34);
5'-CGACGTGCAGAGGTGAA-3' (OLIGO CJP 152: SEQ ID NO: 35);
5'-CGACGTGCAGAGGTGA-3' (OLIGO CJP 153; SEQ ID NO: 36);
5'-CGACGTGCAGAGGTG-3' (OLIGO CJP 154: SEQ ID NO: 37);
5'-CGACGTGCAGAGGT-3' (OLIGO CJP 155: SEQ ID NO: 38);
5'-CGACGTGCAGAGG-3' (OLIGO CJP 156: SEQ ID NO: 39);
5'-CGACGTGCAGAG-3' (OLIGO CJP 157: SEQ ID NO: 40);
5'-AACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 158: SEQ ID NO: 41);
5'-CGACGTGCAGAGGTGAAGCGAAG-3' (OLIGO CJP 159: SEQ ID NO: 42); or
5'-CGACGTGCAGAGGTGAAGCGAA-3' (OLIGO CJP 160: SEQ ID NO: 43).

6. An olignucleotide of Claim 1, the sequence of which consists essentially of one of the following:

5'-CAACGTGCAGAGGTGAAGCGA-3' (OLIGO HBV 10011; SEQ ID NO: 6);
5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);
5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);
5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);
5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);
5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);
5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51); or
5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52).

7. A method of preventing replication of hepatitis B virus (HBV) in a mammalian cell, comprising introducing into the cell an inhibitory amount of an antiviral oligonucleotide of Claim 1.

8. A method of Claim 7, wherein the antiviral oligonucleotide is
5'-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 45),
5'-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 46),
5'-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 47), or
5'-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 48),
or a portion thereof, provided that when said oligonucleotide is a ribonucleotide, "T" in each of said sequences represents "U".

9. A method of Claim 7, wherein the antiviral oligonucleotide consists essentially of a sequence selected from the group consisting of:
5'-CAACGTGCAGAGGTGAAGCGA-3' (HBV 10011; SEQ ID NO: 6);

5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);
5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);
5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);
5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);
5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);
5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51); or
5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52).

10. An expression vector comprising a sequence that is transcribed within a cell to generate an oligonucleotide of Claim 1, said oligonucleotide being an oligoribonucleotide.

11. An expression vector of Claim 10, wherein said vector comprises transcription control sequences that permit it to be expressed within a hepatocyte.

12. An expression vector of Claim 10, wherein said oligoribonucleotide has a sequence corresponding to
5'-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 45),
5'-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 46),
5'-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 47), or
5'-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 48),
or a portion thereof, provided that "T" in each of said sequences represents "U".

13. An expression vector of Claim 10, wherein the sequence of the antiviral oligonucleotide consists essentially of one of the following:

5'-CAACGTGCAGAGGTGAAGCGA-3' (HBV 10011; SEQ ID NO: 6);
5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);
5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);
5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20); 5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);
5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);
5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51); or
5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52),
provided that "T" in each of said sequences represents "U".

14. A method of preventing replication of hepatitis B virus (HBV) in a mammalian cell, comprising introducing into the cell an inhibitory amount of an expression vector of Claim 10.

15. A method of Claim 14, wherein said oligonucleotide has a sequence consisting essentially of
5'-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 45),
5'-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 46),
5'-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 47), or
5'-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 48),
or a portion thereof, provided that "T" in each of said sequences represents "U".

16. A pharmaceutical composition consisting essentially of an amount of an oligonucleotide of Claim 1 effective to inhibit replication of HBV in the liver cells of an animal, and a pharmaceutically acceptable carrier.

17. A pharmaceutical composition of Claim 16, wherein said oligonucleotide has a sequence consisting essentially of
5'-TGGGCGTTCACGGTGGTCGCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 45),
5'-TGGGCGTTCACGGTGGTCTCCATGCAACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 46),
5'-TGGGCGTTCACGGTGGTCGCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 47), or
5'-TGGGCGTTCACGGTGGTCTCCATGCGACGTGCAGAGGTGAAGCGAAG-3' (SEQ ID NO: 48),
or a portion thereof, provided that when said oligonucleotide is a ribonucleotide, "T" in each of said sequences represents "U".

18. A pharmaceutical composition of Claim 17, wherein the sequence of the oligonucleotide consists essentially of one of the following:
5'-CAACGTGCAGAGGTGAAGCGA-3' (OLIGO HBV 10011; SEQ ID NO: 6);
5'-CGACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 114; SEQ ID NO: 10);
5'-ACGTGCAGAGGTGAAGCGA-3' (OLIGO CJP 140; SEQ ID NO: 24);
5'-CGACGTGCAGAGGTGAAGCG-3' (OLIGO CJP 150; SEQ ID NO: 20);
5'-GACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 49);
5'-AACGTGCAGAGGTGAAGCGA-3' (SEQ ID NO: 50);
5'-GACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 51); or
5'-AACGTGCAGAGGTGAAGCG-3' (SEQ ID NO: 52).

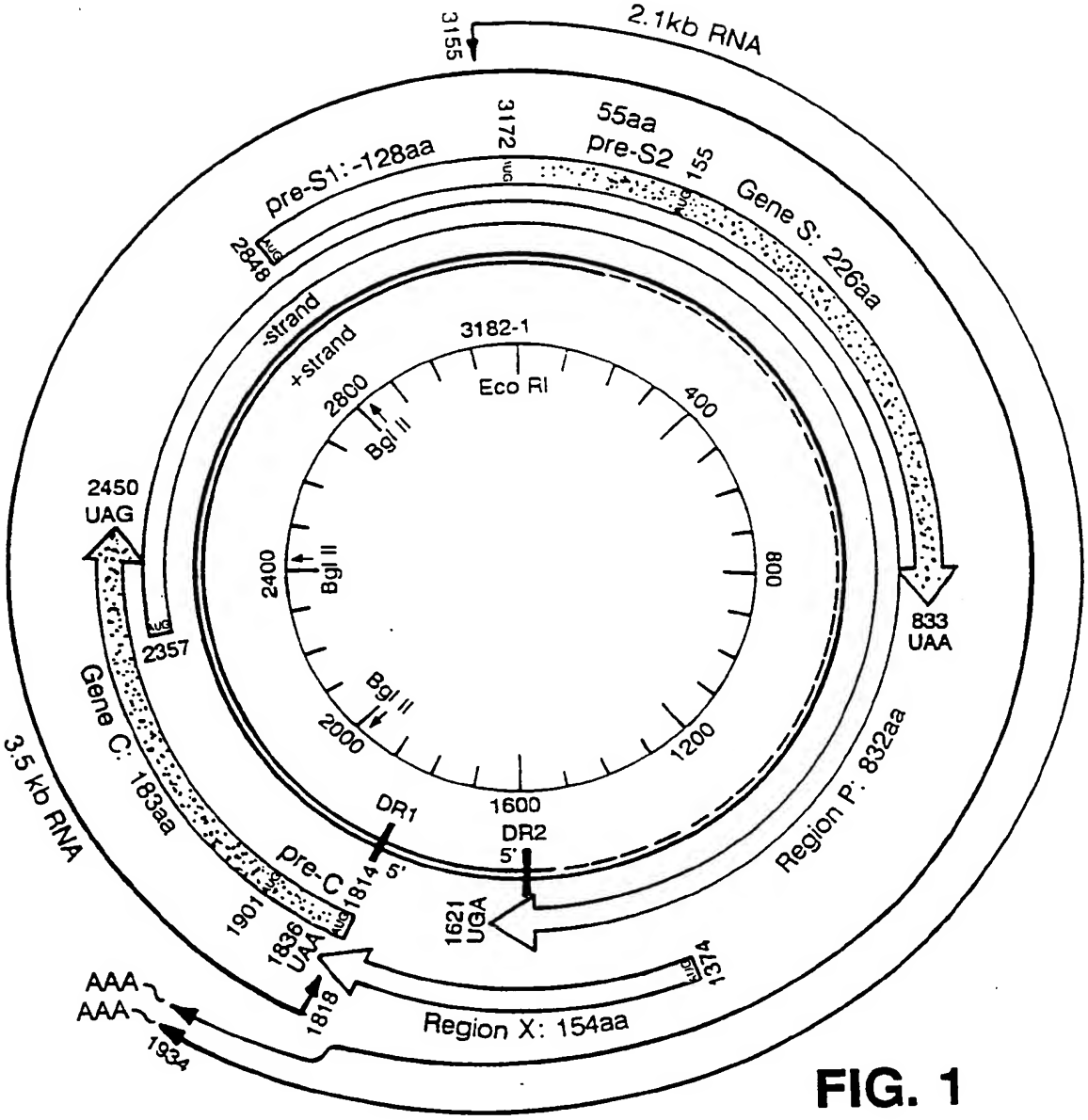
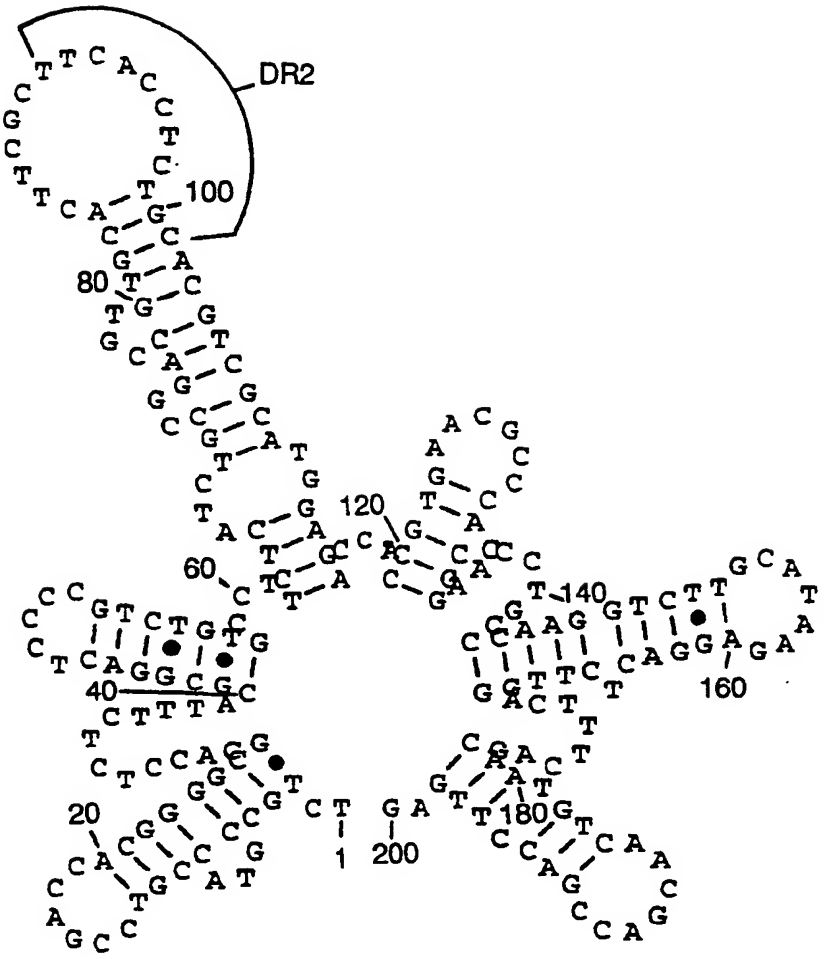


FIG. 1



201 ENERGY = -41.8 HPBADW1 3215 bp
ds-DNA

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00, 21/04; A61K 48/00
US CL :514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, WORLD PATENT INDEX, CA SEARCH; APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Virology, Volume 71, issued 1990, G. Goodarzi et al., "Antisense oligodeoxyribonucleotides inhibit the expresssion of the gene for hepatitis B virus surface antigen," pages 3021-3025, see entire article, especially Table 1.	1-9, 16-18
Y	The Journal of Biological Chemistry, Volume 267, Number 18, issued 25 June 1992, G. Y. Wu et al., "Specific Inhibition of Hepatitis B Viral Gene Expression in Vitro by Targeted Antisense Oligonucleotides, pages 12436-12439, see entire article, especially Materials and Methods.	1-9, 16-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 FEBRUARY 1995	Date of mailing of the international search report 24 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 281, issued 25 October 1979, F. Galibert et al., "Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli," pages 646-650, see entire article.	1-9, 16-18
Y	Nucleic Acids Research, Volume 11, Number 6, issued 1983, Y. Ono et al., "The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw," pages 1747-1757, see entire article.	1-9, 16-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13687

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-9 and 16-18

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-9, 16-18, drawn to a first product, oligonucleotides or pharmaceuticals comprising these oligonucleotides and a first appearing method of use of the product, to prevent replication of hepatitis B virus in mammalian cells.

Group II, Claims 10-13, drawn to a second product, expression vectors for transcribing an oligonucleotide sequence.

Group III, Claims 14-15, drawn to a second method for preventing replication of hepatitis B virus in mammalian cells, by administering expression vectors.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a first appearing product and use of that product, the special technical feature of Group I being the use of oligonucleotides as inhibitors of viral replication. Group II is drawn to a distinct product, expression vectors, which is distinct in form and function from the oligonucleotides of Group I in the use for inhibiting viral replication. The oligonucleotides of Group I are administered directly to cells in order to function, while the vectors of Group II must not only enter the cells, but must then express the inhibitor. Group III is drawn to a second method of preventing viral replication. In addition, PCT Rules 13.1 and 13.2 do not provide for multiple distinct products and methods within a single application.